PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:	
Handfield, et al.	Group Art Unit: 1646
Serial No.: 09/995,493	Examiner: Baskar, P.
Filed: November 28, 2001	Docket No.: 01-662

For: **Identification of *Actinobacillus actinomycetemcomitans* antigens for use in the diagnosis, treatment and monitoring of periodontal disease**

DECLARATION

Commissioner for Patents
PO Box 1450
Alexandria, VA 22313-1450

Dear Sir:

1. I, Dr. Martin Handfield, am a named inventor of U.S. Patent Application Serial No. 09/995,493, filed on November 28, 2001 ("the Patent Application").

2. I have reviewed the Office Action of February 7, 2005 and the references cited therein.

3. Pathogenic microbes can express different proteins, including antigens, under different conditions. For example, *Actinobacillus actinomycetemcomitans* ("*Aa*") may express an antigen under the following conditions:

(a) Under *in vitro* growth conditions only;

(b) Under *in vivo* growth conditions only; or

(c) Under *in vitro* and *in vivo* growth conditions.

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IVIAT methodology can identify antigens that are expressed by *Aa* under *in vivo* conditions only (*i.e.*, condition (b) above). See specification page 9, first and second full paragraphs. This is important because there are antigens that are expressed only when *Aa* is engaged in actually causing disease in, *e.g.*, humans. Important environmental signals that normally cause *Aa* to turn on virulence genes during an infection are missing when the bacteria are grown *in vitro*. Therefore, many of the best targets for diagnostic and vaccine strategies are unknown. IVIAT methodology identifies antigen that are specifically turned on during growth of *Aa* in a human host and not during routine *in vitro* laboratory growth. These antigens are useful in developing diagnostic tests for *Aa* to identify, for example, subjects who are in early stages of infection and for monitoring response to therapy, and for developing vaccines or treatments to prevent or treat diseases caused by *Aa* in susceptible animals. *See* specification, page 9, line 14 through page 10, line 1.

4. Therefore, antigens discovered using the IVIAT method are only expressed under *in vivo* conditions (condition (b) in paragraph 3, above) and would not be expected to be expressed in *Aa* cultures grown under *in vitro* conditions.

5. Flemmig *et al.* teaches methods of detecting the presence of anti-*Aa* antibodies in a test sample using outer membrane proteins (OMPs) of ***in vitro* grown** cultures of *Aa*. *See* Flemmig, page 678, col. 2., third and fourth full paragraphs. The OMPs are used to determine antibody reactivity of human sera to OMPs. Therefore, Flemmig is detecting antibodies in human sera that specifically bind to ***in vitro* grown** OMPs of *Aa*.

6. Antibodies or fragments thereof that specifically bind a purified immunogenic polypeptide comprising SEQ ID NO:52 would not specifically bind to *in vitro* grown

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OMPs of Flemmig because the immunogenic polypeptide of SEQ ID NO:52 is not expressed under *in vitro* growth conditions. Since SEQ ID NO:52 is not expressed *in vitro* it would not be present in the *in vitro* grown OMPs of Flemmig.

7. One of skill in the art would not expect an *in vivo* only expressed polypeptide identified by IVIAT technology to be among the *in vitro* expressed OMPs of Flemmig. Therefore, SEQ ID NO:52 and fragments thereof would not be present among the ***in vitro*-expressed** OMPs that are used by Flemmig to detect anti-*Aa* antibodies in human sera. As such, Flemmig cannot anticipate the subject matter of claims 32, 33, 36, 37, 40 and 41.

8. Ebersole teaches the detection of anti-*Aa* antibodies in test samples by contacting the test sample with ***in vitro* grown** *Aa* outer membrane antigens (OMAs). Ebersole teaches OMAs of *Aa* that are isolated from ***in vitro* grown cultures**. See Ebersole, page 659, second col., first and fourth full paragraphs. Ebersole, however, cannot teach or suggest the claimed polypeptides because Ebersole teaches the use of OMAs that are **expressed *in vitro*** (and possibly expressed both *in vitro* and *in vivo*). The polypeptide of SEQ NO:52 was identified using IVIAT methodology and is therefore expressed only *in vivo*. A polypeptide of SEQ ID NO:52 cannot be present in the OMAs used in Ebersole because they are expressed only *in vivo* and would not be present in the ***in vitro* grown cultures** of Ebersole. Therefore, Ebersole cannot anticipate the subject matter of claims 32, 33, 36, 37, 40, and 41.

9. The Snyder references (Snyder *et al.* EP 0439210 (Snyder '210); EP 0439211 (Snyder '211); EP 0439212 (Snyder '212) or Snyder EP 537830 (Snyder '830)) teach that the polyclonal antibodies used in the disclosed methods are generated by injecting

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rabbits with whole ***in vitro*** **grown** cultures of *Aa*. See Snyder ('212) page 10, Col. 15, lines 10 through 45; Snyder ('210) page 9, lines 3-49; Snyder ('211) page 3, Col. 3, line 55 through Col. 4, line 24. Therefore, the Snyder antibodies are specific for and bind to *Aa* antigens that are present in ***in vitro*** **grown cultures** of *Aa*. In contrast, the claimed antibodies bind SEQ ID NO:52, which is expressed only *in vivo*. The antibodies used in the instant invention are specific for and specifically bind a polypeptide that is **expressed *in vivo* only**, while the antibodies of the Snyder references are specific for and specifically bind polypeptides or antigens that are expressed *in vitro* or expressed *in vivo* and *in vitro* (which is different from expression *in vivo* only). Since a polypeptide of SEQ ID NO:52 is expressed *in vivo* only, it would not have been present in the preparation of *in vitro* grown *Aa* polypeptides that were used to immunize the rabbits of Snyder. Therefore, the antibodies of Snyder could not specifically bind to a purified immunogenic polypeptide comprising SEQ ID NO:52 as recited by the instant claims, because a polypeptide of SEQ ID NO:52 was not present in the immunizing composition.

10. I hereby certify that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patents issued thereon.

Date: 5/2/2005 By: _____

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In vivo induced genes in human diseases

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The quest for *in vivo* induced genes: a historical perspective

Microorganisms are very good at sensing their environment and adjusting the expression of their genes to accommodate changes that they sense. One fundamental feature of microbial infections is that they are multifaceted and dynamic processes. The site of infection undergoes constant change from the moment a pathogen enters the human host. In most instances, expression of the pathogen's virulence determinants is tightly regulated in response to the changing environment encountered at the site of infection (12, 16, 34). For this reason, it is extremely unlikely that all virulence determinants of a human pathogen could be identified simply by studying the pathogen in the laboratory since it would be technically impossible to determine and mimic all of the complex and changing environmental stimuli that occur at the site of an infection. This shortcoming hampers our complete understanding of the virulence mechanisms employed by human pathogens (12, 34, 42) and is reflected in the relatively small number of effective vaccines that are currently available to combat the myriad of infections that afflict mankind. To overcome this problem, a number of investigators have emphasized the need to study bacterial virulence using organisms engaged in an actual infectious process. The ultimate goal is to identify those genes that are specifically expressed during infection, so-called *in vivo* induced (IVI) genes. Such genes and their expressed proteins are very likely to be directly involved in the organism's ability to cause disease or promote its survival in the host. IVI genes therefore are likely to serve as excellent targets for the development of new vaccine, diagnostic, and antibiotherapy strategies.

During the past 10 years, a number of novel genetic, microarray, and proteomics approaches have been developed that have greatly increased our ability to identify bacterial IVI genes. Methods such as *in vivo* expression technology (32), differential fluorescence induction (45, 46), and signature-tagged mutagenesis (22) have yielded valuable information regarding pathogenic mechanisms of certain microorganisms. For example, pathogenicity islands were characterized with these new tools (21), the global role for DNA adenine methylation in virulence was established (20), and new targets for antibiotic therapy were initially discovered from the study of IVI genes and proteins (45). In *Salmonella typhimurium* and *Pseudomonas aeruginosa*, for example, mutant analysis has been used to confirm that certain genes identified by these methods do, in fact, encode virulence factors as assayed in animal models of infection (16, 17, 20, 32).

The various technologies presented above have significantly contributed to our understanding of bacterial virulence. However, all of these approaches suffer from one or more important drawback, which are summarized in Table 1. Their common and most severe limitation is their reliance on animal models of infection. In many instances, animal models are not available. In many other cases, the animal models do not closely resemble the conditions found within the natural human host, or they do not use the natural route of infection of the pathogen. A number of examples can be found in the literature of erroneous conclusions being drawn by extrapolation of results from animal models to humans (42). Many of these technologies are not readily applicable to genetically 'undomesticated' microorganisms, which are microorganisms lacking a well established or reliable means for genetic manipulation. With the sole exception of a modified *in vivo* expression technology approach (RIVET; 7), which was technically difficult to perform, none of these methods is very

Table 1. Advantages and limitations of selected *in vivo* expression technologies

System	Relevant characteristics	Limitations
Reporter genes (*lacZ*, GFP, *pho*A, CAT)	Screening for differentially regulated genes.	Brute force screening which requires an animal or *in vitro* model, and a genetically domesticated pathogen.
IVET (auxotrophic or antibiotic resistance)	Promoter trap or probe which selects for up-regulated genes.	Requires an animal or *in vitro* model, and a genetically domesticated pathogen.
DFI	FACS-based screening.	Requires an animal or *in vitro* model, and a genetically domesticated pathogen.
STM	Comparative hybridization.	Requires an animal or *in vitro* model, and a genetically domesticated pathogen.
Differential display	Multiplex comparative hybridization.	Requires an animal or *in vitro* model, and sufficient high quality mRNA. Assess transcriptional level of regulation only.
Microarrays	Multiplex comparative hybridization.	Requires an animal or *in vitro* model, sufficient high quality mRNA, and a sequenced genome. Technically demanding. Assess transcriptional level of regulation only.
Proteomics and 2-D gels	Screen for differentially regulated proteins.	Amendable to relatively simple *ex vivo* systems. Technically demanding. Low sensitivity.
IVIAT	Identifies immunogenic factors during actual human infection.	Requires a cultivable pathogen and serum from patients.

IVET, *In vivo* expression technology. DFI, differential fluorescence induction. STM, signature-tagged mutagenesis.

well suited to identify IVI genes that are transiently expressed during the course of an infection. Finally, these methods are generally restricted to the use of a single representative strain of the pathogen since, from a technical standpoint, the use of multiple strains is very demanding. This restriction can be particularly important in instances where a pathogenic species demonstrates strain-specific differences in pathogenic potential (so-called *clonality*).

We ultimately were successful in developing an *in vivo* expression technology strategy for identification of IVI genes in *Porphyromonas gingivalis* (29, 48). However, we were unable to utilize this or other existing technologies to analyze *Actinobacillus actinomycetemcomitans* because of a lack of a suitable animal model and because, at the time, genetic manipulations in this species were unreliable at best. We therefore devised a completely novel approach for identifying IVI genes, which was termed *In Vivo* Induced Antigen Technology (IVIAT; 15, 18). Proof of principle for IVIAT was accomplished using *A. actinomycetemcomitans* infection in patients suffering from localized aggressive periodontitis. The successful identification of IVI genes in this pathogen, without the use of animal models and without the need for genetic manipulations in *A. actinomycetemcomitans*, was accomplished and has led to the widespread adoption of this method for the identification of IVI genes.

In Vivo Induced Antigen Technology

A general outline of IVIAT is presented in Fig. 1. First, sera from infected and/or convalescent patients are pooled and adsorbed with whole cells and cell extracts of the pathogen grown *in vitro* (**A**). This step removes antibodies present in the serum that are reactive with proteins that are constitutively expressed by the pathogen. Antibodies that remain in this adsorbed serum are reactive with proteins that are specifically expressed by the pathogen during the natural infection. At the same time that the adsorbed serum is being prepared, genomic and/or plasmid DNA is purified from cultures of the pathogen. This genetic material is used to construct an inducible expression library, which is propagated in *Escherichia coli* or other suitable host (**B**). Samples of the library are probed, typically using a colony lift format, with

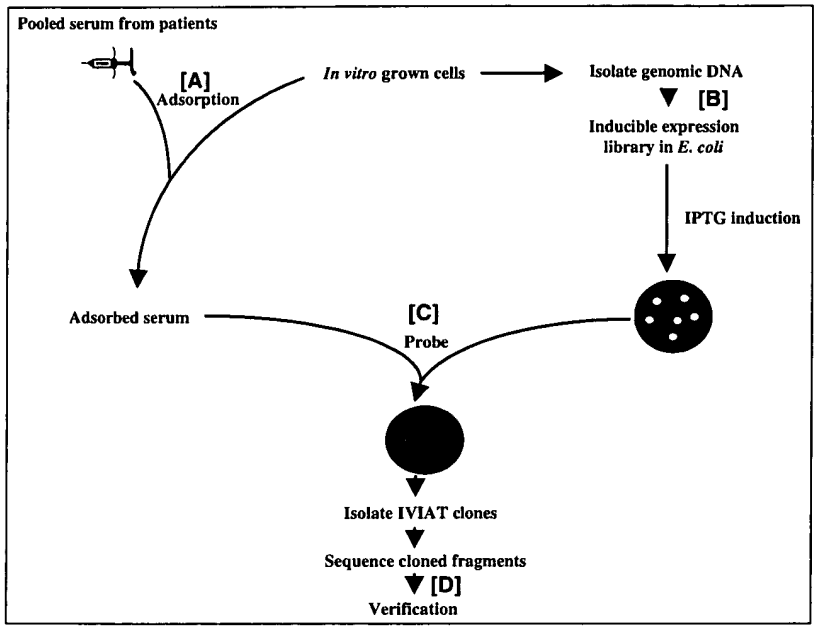

Fig. 1. ***In vivo*** **induced antigen technology (IVIAT).**

the adsorbed serum (**C**). Clones that are immunoreactive with the adsorbed serum contain a fragment of the pathogen's DNA that expresses an IVI gene, thereby producing a protein of the pathogen that is specifically produced during a natural infection, but which is not expressed under conditions of *in vitro* cultivation. Clones expressing IVI antigens are purified and their cloned DNA sequenced. Independent methods, such as immunofluorescence microscopy or real time polymerase chain reaction (PCR) can then be used to independently confirm that the IVI gene products are indeed specifically expressed during active disease (**D**) (15, 18).

A few details are worth mentioning regarding the serum for use in an IVIAT project. First, serum can be pooled from a number of patients with a clear-cut diagnosis of the disease being studied. The samples should also be taken from patients at different time points in the infectious process. This approach logically serves to maximize the spectrum of antibodies directed against IVI antigens produced only transiently in the infectious process, and that may vary between individuals. In instances where the pathogen manifests disease via different routes of infection (*Streptococcus-pyogenes* induced pharyngitis, fasciitis, impetigo, etc.), serum from patients representative of each of the routes will provide the opportunity to identify IVI genes that are route-specific.

In a similar fashion, certain details regarding the pathogen should be considered before undertaking an IVIAT analysis. When the pathogen of interest demonstrates clonality, where certain strains (clonotypes) are more pathogenic than others, clearly the choice of starting strains is extremely important. If this information is not thoroughly established, IVIAT has the advantage of being able to analyze several different clonotypes simultaneously. This is achieved by creating the genomic expression library using DNA from two or more strains. Also, certain microorganisms may provide a potential source of difficulty through the production of immunoglobulin degrading proteases, or immunoglobulin-binding proteins. In these cases, pretreatment of the cells may be required to prevent degradation or loss of the serum antibodies during the adsorption steps.

Several lessons have been learned from using IVIAT with different prokaryotic and eukaryotic systems over the past 5 years. To present them with the emphasis they deserve, they are itemized below:

1. Of the microorganisms studied thus far, it is clear that humans respond to a very broad array of proteins expressed by the pathogen. This appears to be the case regardless of whether the antibodies serve a protective role or are just 'bystander' antibodies (26). Thus, contrary to the *a priori* feelings that have been expressed by different sources, the human humoral response is directed against much more than just surface proteins of the pathogen. This enables IVIAT to identify a broad array of IVI genes, regardless of the cellular localization of their expressed products.
2. An IVI gene is not necessarily a virulence gene, nor is it a foregone conclusion that it is absolutely indispensable for survival *in vivo*. In the case of both vaccine and diagnostic strategies, neither of these attributes is essential, although in the former

case it might be highly preferable. Thus, the sole purpose of IVIAT is to identify IVI genes that are *more likely* than *in vitro* and constitutively expressed genes to be important to the microorganism's pathogenic personality and that would probably not be found using conventional methods. Once the IVI genes of a pathogen have been identified, conventional biochemical, genetic, and/or immunological methods must be applied to prioritize them with regard to the ultimate goals of the project.

3. As in the case of all other *in vivo* expression technologies, IVIAT will not identify every virulence factor expressed by a particular pathogen. Obviously, it would not identify those virulence factors that are expressed during *in vitro* as well as *in vivo* growth. These, we presume, will be discovered using conventional *in vitro* methods. Also, there may be proteins that, for whatever reason, are not immunogenic. Clearly, however, IVI genes that IVIAT does identify would almost certainly not be found by conventional *in vitro* biochemical, genetic or immunological methods. And, because IVIAT does not depend on animal models of infection, and because of its speed and ease of application to any microorganism, its ability to identify transiently expressed genes, and its flexibility with regard to potential clonality problems, IVIAT is significantly superior to other reported *in vivo* expression technologies.
4. One of the truly remarkable aspects of IVIAT is its simplicity. Years of experience in growing and working with a particular pathogen are not required to successfully accomplish the screening aspects that lead to identification of IVI genes.
5. The *in vitro* cultivation conditions used to grow cells of the pathogen for serum adsorption is an important consideration for IVIAT. As in the case of a pathogen growing *in vivo*, expression of certain genes during *in vitro* growth is regulated by conditions such as cultivation medium, atmosphere, pH, and growth phase. The use of minimal medium logically results in a reduction of the number of genes involved in central intermediary metabolism isolated by IVIAT, and increases the number of transport proteins found. The use of rich medium gives the opposite result. Some basic knowledge of the environment at the site of infection may help decide the best cultivation conditions to use for a particular IVIAT study.
6. We also do not assume that any gene is totally shut off under any *in vitro* growth condition, since there may be a basal level of expression. Our experience with IVIAT indicates that, while our adsorption process may possibly exhaust the sera of antibodies directed at certain differentially expressed genes, this is not the general case. Through trial and error, our adsorption process has been refined to achieve an excellent balance between eliminating antibodies directed against *in vitro* induced antigens and preserving antibodies directed against IVI antigens. Taking into account **3** above, the fact that we have already identified a significant number of interesting IVI genes from a broad array of human pathogens is the strongest argument that IVIAT works and yields desirable results.
7. Finally, antibody probes from infected animals or humans have been used in the past to study bacterial virulence (e.g. 1, 30, 31, 49). Because the targets of these probes were cells grown *in vitro*, these studies were designed to identify genes that were constitutively expressed or regulated genes that happened to be expressed under the conditions of *in vitro* cultivation that were used. IVIAT is clearly and expressly different from these sorts of studies. By adsorbing out antibodies directed against *in vitro* expressed genes and by probing a genomic expression library of the pathogen's DNA, IVIAT identifies genes of the pathogen that are specifically expressed *in vivo*, and in particular, during infection of an actual human host. There is currently no other technology that can achieve this end.

The IVIAT prototype: *A. actinomycetemcomitans*

A. actinomycetemcomitans was the prototype microorganism used to develop IVIAT. It is the etiological agent of localized aggressive periodontitis (14, 41, 50). The pathogenicity of *A. actinomycetemcomitans* is multifaceted and the pathophysiology of the infection is influenced by both microbial and host determinants. A number of putative virulence factors have been described for this microorganism (reviewed in 13). *A. actinomycetemcomitans* invades epithelial tissues and produces several biologically active substances that collectively could be involved in disease causation and persistence.

Prior to IVIAT, it had already been shown that a number of putative *A. actinomycetemcomitans* virulence factors were indeed regulated by environmental stimuli. For example, an *A. actinomycetemcomitans* hemolysin is regulated by environmental iron

concentrations (23). Production of the *A. actinomycetemcomitans* leukotoxin also appears to be affected by a number of environmental stimuli, including oxygen tension (24, 44), fermentable sugar levels (35), and bicarbonate concentrations (37). Furthermore, the environmental iron concentration has been reported to regulate the level of production of certain outer membrane proteins in *A. actinomycetemcomitans*, some of which are likely to be associated with its virulence (40). Altogether, these studies clearly demonstrated that crucial virulence-associated factors of *A. actinomycetemcomitans* are regulated through environmental signals, and emphasized the need to use an *in vivo* technology, in particular IVIAT, to identify other virulence genes that would not be found simply by manipulating cultivation conditions.

The genome-wide IVIAT screening of *A. actinomycetemcomitans* strain HK1651 was performed. This entailed using adsorbed serum from 20 patients to probe 300,000 clones of an expression library constructed using HK1651 genomic DNA. A total of 116 IVI genes and their expressed antigens were found (43). IVIAT identified antigens relevant to pathogenesis, but which would be relatively difficult or even impossible to identify using alternative techniques. Computational methods, such as those available in the Pedant-Pro Sequence Analysis Suite (ALOM2, InterProScan, NCBI-BLAST, ProSearch, SignalP, and PSORT) allowed us to classify most *in vivo* induced (IVI) genes of *A. actinomycetemcomitans* into functional categories. These categories include functions that are associated with nutrient acquisition, stress response and adaptation, response to environmental stimuli, gene regulation and intermediary metabolism (43). In addition, a significant number of IVI genes that were recovered encode conserved hypothetical proteins that have no known function. These genes provide a particularly interesting group from the standpoint of furthering our overall knowledge of *A. actinomycetemcomitans* pathogenicity and for serving as potential targets in vaccine and diagnostic strategies. In this regard, a large proportion of *in vivo* induced antigens either presented evidence of association to host–pathogen interaction or were surface-localized. A total of 32 IVI genes were found in functional classes involved with microbe–host cell interaction, colonization and/or bacterial cell surface structures. As presented in Table 2, their high degree of homology to known virulence determinants in other pathogens and/or their cellular localization suggests that these gene products have a significant probability of being directly involved in pathogenesis. Numerous examples of IVI genes presenting the same characteristics have been previously found in other organisms and have been directly associated with host cell interactions and pathogenesis. These genes include *PilA* and *TcpA* of *Vibrio cholerae* (19), *HlyU* of *Vibrio vulnificus* (27), and the LEE-encoded *intimin-γ* from *E. coli* O157:H7 (33), to cite only a few of the most significant examples.

IVI proteins, by definition, are not produced under growth conditions that are usually used in the laboratory. This significantly hampers our capability to study their function and role in pathogenesis under controlled laboratory conditions. Nonetheless, we previously reported using immunofluorescent-labeled antibodies that one IVI antigen, the product of orf859, was expressed by *A. actinomycetemcomitans* in dental plaque of localized aggressive periodontitis patients but not during *in vitro* cultivation (15, 18). Although immunofluorescence presented the most direct and definitive demonstration of *in vivo* induction of orf859, it was too cumbersome an approach to use for the routine validation of other IVIAT-identified antigens, or to measure their induction under different, modified environmental conditions. This led us to develop a real-time PCR approach for this purpose, which has the advantages of being rapid, semiquantitative and convenient. Of the 32 novel *A. actinomycetemcomitans* antigens mentioned above, eight thus far have been tested for their transcriptional induction upon adhesion and/or invasion of epithelial KB (HeLa) cells and human immortalized gingival keratinocytes (HIGK) cells (38). These cell lines provided us with a convenient and relevant means to test the putative function of IVI genes and their relevance to virulence under controlled conditions in the laboratory. In this initial step, we were careful in choosing genes for further characterization that would represent IVIAT genes of both known and unknown functions. The results presented above allowed us to propose that IVIAT can be used to validate *in vitro* model systems, cell lines or animal models of infection. The rationale of this hypothesis is based on the identification of experimental models that lead to the same bacterial gene expression pattern as those observed with IVIAT in human infections.

The *A. actinomycetemcomitans* genes characterized so far can be studied further by conventional genetic, biochemical and immunological techniques with the *a priori* confidence of their relevance in human infections. For example, of the eight antigens used for the reverse-transcription polymerase chain

Table 2. Characteristics of selected *in vivo* induced *A. actinomycetemcomitans* antigens

Annotation[a]	Putative function[a]/relevant characteristic(s)[b]
ORF859 (Hi ORF0701)	Uncharacterized conserved hypothetical protein. This gene is induced in HIGK and HeLa cells. LJP patients have a significantly ($P < 0.05$) greater anti-orf859 antibody titer than sera from healthy controls. Surface-localization confirmed by immunofluorescence. Mutated strain JMS04 (orf859-) is defective in intracellular fitness as compared to wild-type in a competition assay. Last of a four-gene operon (8, 37).
ORF1402 (Hi OMP1)	Outer-membrane protein involved in long-chain fatty acid transport. This gene is induced in HIGK and HeLa cells. LJP patients have a significantly ($P < 0.05$) greater anti-orf1402 antibody titer than sera from healthy controls. Mutated strain JMS05 (orf1402-) is defective in intracellular fitness as compared to the wild-type in a competition assay. Monocistronic (8, 37).
ORF604 (Bs estA1)	Putative acetylcholinesterase or type B carboxylesterase involved in virulence in *B. subtilis.*
ORF871 (Hi ORF0874)	Uncharacterized protein family. Related to lipid A core-O-antigen ligase. Presents 11 transmembrane regions. Fourth gene of a five-gene operon.
ORF1083 (Pm DegS)	Predicted membrane-associated Zn-dependent proteases involved in virulence in *B. subtilis*, and heat shock response in *H. pylori.*
ORF1180 (Pm MsbA)	Membrane type I protein secretion system (ABC-type transport systems). Presents 4 transmembrane regions. Second gene of a five-gene operon.
ORF1300 (Hi NlpC-like)	Hypothetical protein HI1314 of *Haemophilus influenzae.* Cell wall-associated hydrolase involved with invasion.
ORF1623 (Aa ORF4)	Putative membrane glycosyltransferase involved in serotype-specific polysaccharide biogenesis. Large polycistronic operon.
ORF1766 (Pm 0351)	GTP binding, stress response and virulence in *Listeria monocytogenes.*
ORF 1795 (Hi hel)	Predicted secreted acid phosphatase and membrane lipoprotein.

[a]Annotation in the PEDANT database (http://pedant.gsf.de/) as of November 2004. Closest homolog using BLAST at NCBI. Aa, *A. actinomycetemcomitans*; Bs, *Bacillus subtilis*; Hi, *Haemophilus influenzae*; Pm, *Pasteurella multocida*; LJP, localized juvenile periodontitis.
[b]Characteristics deduced from results presented in references 8 and 37.

reaction (RT-PCR) analysis mentioned above, three were chosen for further mutant and phenotypical analysis. The adhesion and intracellular survival of the mutants were assayed in a competition assay with the wild-type strain. A significant defect in the intracellular survival of two of these mutant strains (orf1402 and orf859) was found. This defect could not be attributed to an adhesion defect. In contrast, a mutation in *vap*A, a homolog of a novel putative transcriptional regulator, out-competed the wild-type strain in the same assay. The virulent phenotype was restored for a mutant strain in orf859 upon complementation (8).

At present, radiographic evidence of bone loss is the only available diagnostic tool for *Actinobacillus*-related periodontal diseases. However, this method's lack of sensitivity means that by the time disease is detected, it has progressed to the point where irreparable damage has occurred. As is the case with a number of other human pathogens, current detection methods cannot distinguish between the colonization state vs. the active and destructive disease state. One of the most interesting outcomes of the *A. actinomycetemcomitans* IVIAT screen is the finding of new and novel putative diagnostic targets. As mentioned above, this microorganism is a member of the human indigenous flora. Hence, it was expected that most subjects would have circulating antibodies directed against proteins of *A. actinomycetemcomitans* that are associated with persistent colonization. With the onset of periodontitis, and in response to the changing environment at the site of infection, we speculated that new bacterial proteins would be produced, and that these products could be associated with the initiation and progression of the disease state. It is this latter group of proteins that were particularly interesting from the standpoint of their potential to serve as targets for diagnostic and therapeutic strategies.

A Western blotting approach was thus used to determine whether sera from periodontitis patients possessed higher reactivity against IVIAT proteins

than sera from healthy control patients. A significant titer was found in healthy controls for all antigens tested to date. This observation was consistent with the status of *A. actinomycetemcomitans* as a member of the normal oral flora, or the result of cross-reactivity with other oral species. However, for six of the nine antigens tested, sera from patients had significantly higher antibody titers than sera from healthy controls, which supports their potential usefulness as disease markers (8). These data also suggested that these six antigens were broadly immunogenic, although some patient-to-patient differences were observed in their levels of reactivity. It was concluded that the expression of those six antigens may be particularly relevant to the disease state, and attractive targets for the development of novel diagnostic tools. The other three IVI antigens tested, which showed a level of reactivity similar to the level of reactivity in control subjects, were postulated to have a role in colonization rather than in disease causation.

Other oral and nonoral human pathogens

Thus far, a number of groups have utilized IVIAT to study the pathogenesis of various eukaryotic and prokaryotic mammalian pathogens. A summary of selected projects is presented below.

Porphyromonas gingivalis

The *P. gingivalis* genome was the second genome that was entirely surveyed by IVIAT for IVI genes, and generated over 100 genes of interest. The pattern of genes recovered for further analysis was very similar to the pattern found in *A. actinomycetemcomitans* (43). Interestingly, a number of these genes were also previously reported as being *in vivo* induced using *in vivo* expression technology (29, 48). Isogenic mutants of at least one of these genes (*ivil0*, homologous to a putative TonB-dependent outer membrane receptor protein) have already been generated and have shown significantly decreased virulence when a mouse model was challenged with this mutant (48). Based on their homology to known virulence factors, many of the recovered genes are likely to be important in the virulence of this microorganism. In addition, 40% of the recovered genes have no homology in the databases. Such genes are likely candidates to serve as highly specific diagnostic and therapeutic targets (43).

Candida albicans

Candida albicans is the most common fungal pathogen of humans. Normally, this organism is commonly associated with mucosal surfaces, and lives in a commensal relationship with the human host (39). If the host becomes immunodepressed or immunocompromised, or if alterations occur in the normal microbial flora, *C. albicans* is capable of causing a wide range of diseases by invading and damaging underlying tissues. Several virulence determinants have been identified in *C. albicans*, including adherence to host cells, secretion of hydrolytic enzymes (proteases and phospholipase), sequestration of iron, survival within phagocytes, yeast-hyphal morphogenesis, and phenotypic switching (5, 6). To date, using IVIAT, collaborators identified a total of 59 *C. albicans* genes induced during human candidiasis (9, 36). Overall, 47 IVI genes of known or putative function can be divided into the following classes (1): transcriptional regulation (16 genes), nutrient synthesis and metabolism (8 genes), cytoskeleton and cell wall structure, organization and biosynthesis (7 genes), stress response and adaptation (7 genes), transport (4 genes), and others (5 genes) (reviewed in 36). The remaining genes are unique to *C. albicans*, with no apparent homologs or identifiable domains in other microorganisms. In their first *C. albicans* publication, Cheng et al. (9) described the first 10 *C. albicans* genes identified with IVIAT. These genes were confirmed by RT-PCR to be expressed at significantly higher levels by cells in thrush pseudomembranes as compared to cells grown *in vitro*. Furthermore, an isogenic mutant for one of the IVI genes (*NOT5*) was shown to be essential for virulence using a murine disseminated candidiasis model of infection (9). This work has provided significant support for the hypothesis that transitions between yeast and hyphal morphologies are essential to *C. albicans* in order to optimize its survival *in vivo*.

Vibrio cholerae and *Vibrio vulnificus*

One of the more fascinating discoveries to date stemming from IVIAT originates from the partial screening of the *V. cholerae* genome (19). This work identified a total of 12 different genes involved in type IV pili expression, toxin production, chemotaxis, and motility. None of these genes was expressed under laboratory culture conditions as demonstrated by RT-PCR. Mutations in many, but not all, of these IVI genes showed defects in virulence in a mouse model of cholera. Of particular interest, PilA, the structural

subunit of a third type IV pilus in *V. cholerae,* was identified in that work. This gene product was originally characterized as a cryptic gene based on genomic information. Interestingly, PilA was shown to be expressed and immunogenic during human infection, but a mutation of *pilA* had no effect on colonization of infant mice by *V. cholerae.* This raised the possibility that PilA may play a specific role in colonization of human intestine and may reflect the limitations of animal models to identify and test the virulence genes associated with human infection. It was also found that all of the genes from chromosome II of *V. cholerae,* identified as being expressed during human infection by IVIAT, were expressed poorly in human stool using microarray technology (4). This result suggested that IVIAT identified transiently expressed genes that were repressed during the organism's passage through the large bowel, making them inaccessible to identification by microarray technology. The fact that convalescent sera from cholera patients specifically recognized PilA further suggested that this pilus may be uniquely expressed during human infection, and may play a role in *V. cholerae* pathogenesis, a finding not suspected by previous animal model experiments or genomic surveys of the pathogen.

V. vulnificus is a remote cousin of *V. cholerae* and the etiological agent of a form of shellfish-acquired septicemia characterized by rapid progression and a fatality rate above 50% in humans. In their study, Kim et al. (27) found 12 IVI genes presenting homologies to factors of diverse functions. Similar to the *V. cholerae* project, one of the recovered IVI genes expressed a chemotaxis-associated factor (a methyl-accepting chemotaxis protein). In addition, other IVI proteins were found, including ones involved in signaling (a GGDEF-containing protein and a putative serine/threonine kinase), biosynthesis and metabolism (PyrH, PurH, and IlvC), secretion (TatB and plasmid *Achromobacter* secretion factor), transcriptional activation (IlvY and HlyU), and the activity of a putative lipoprotein (YaeC), in addition to one open reading frame encoding a hypothetical protein. Isogenic mutant constructions were performed on all of these genes and tested for a decrease in cytotoxicity. Cytotoxic activity of the mutant strains, as measured by lactate dehydrogenase release from HeLa cells, was nearly abolished in *pyr*H, *pur*H, and *hlyU* mutants as compared to the wild-type strain. The intraperitoneal 50% lethal dose in mice increased by approximately 10–50-fold in these three mutants. Although PyrH and PurH appeared to be essential for *in vivo* growth, HlyU appeared, in contrast, to be one of the master regulators of *in vivo* virulence expression in *V. vulnificus* (27).

Mycobacterium tuberculosis

Tuberculosis has reemerged in recent years as the foremost infectious cause of death worldwide: about 8 million new cases of tuberculosis occur annually, resulting in approximately 2–3 million deaths. *M. tuberculosis* infects approximately a third of the world's population. The World Health Organization (WHO) estimated that, based on current rates, up to a half billion people will contract tuberculosis in the next 50 years and that more than 200 million of them will die from it. The reemergence of tuberculosis has depended on a variety of factors, chief among which are the emergence of AIDS, the low efficiency of the BCG vaccine against pulmonary tuberculosis, worsening social conditions, and resistance of up to 15% of clinical isolates to one or more front-line antituberculosis drugs (WHO, 1997). Using a λgt11 variant of IVIAT, *M. tuberculosis* H37Rv has been screened with pooled TB patients' sera. This endeavor led to the identification of six IVI antigens, which could be ascribed functions including probable regulatory proteins, various intermediate metabolism enzymes, and proteins of hypothetical function (11). Recent work using traditional IVIAT methodology has identified an additional 39 putative IVI genes (Hillman et al., unpublished). These genes are currently undergoing further analyses to identify those that will best serve for new diagnostic and vaccine strategies.

Escherichia coli O157:H7

Enterohemorrhagic *E. coli* O157:H7 is a human opportunistic pathogen which causes diseases ranging from acute, self-resolving watery diarrhea to hemorrhagic colitis and the potentially fatal hemolytic uremic syndrome (HUS). To date, the main impediment to the identification of the entire complement of virulence factors of this pathogen has been the unavailability of convenient animal models that mimic all aspects of human disease. Working with four pediatric patients who had recovered from HUS, Manohar et al. recently identified 223 IVI antigens of *E. coli* using IVIAT. Reactive clones mapped to both O157-specific and *E. coli* backbone sequences, which are sequences present in the wild-type *E. coli* and common to all pathogenic and nonpathogenic strains. As with other IVIAT projects, these IVI antigens could be distributed into diverse

functional classes based on analyses of homology and structural motifs. In addition to a number of IVI antigens found using other methods, a number of previously unidentified IVI antigens were identified in this study, thereby clearly demonstrating that IVIAT does indeed find antigens that may not be found using more conventional methods. Of the 42 IVI-antigens found in O157, 13 were unique to O157 and 29 were shared between O157 and 055:H7, the ancestral enteropathogenic *E. coli* strain. Equally significant, two IVI antigens were mapped to the O-island and part of cryptic prophages (33).

Pseudomonas aeruginosa

The IVIAT survey of the *P. aeruginosa* 6.2 Mb genome in cystic fibrosis patients is underway. Over 220 different genes have been identified thus far and are being further characterized for diagnostic and therapeutic applications. Until now, a least one of the known genes that was recovered was a known virulence factor associated with iron scavenging and had been previously identified using *in vivo* expression technology. In the latest report, an isogenic mutant of this gene was created and showed a decreased virulence in a rat chronic lung infection model (25). Of interest, 50% of the genes recovered in *P. aeruginosa* had no orthologs in the databases, which is indicative that our level of understanding of the pathogenesis of the organism is still very incomplete. Consequently, these novel genes may be extremely valuable for the development of novel therapies or prevention measures (Hillman et al., unpublished).

Group A *Streptococcus*

Group A *Streptococcus* causes a variety of human diseases. Some of the best known examples are pharyngitis, impetigo, and some life-threatening invasive diseases such as necrotizing fasciitis and streptococcal toxic shock syndrome (2). Complications of group A *Streptococcus* infections can also lead to sequelae diseases such as rheumatic fever and glomerulonephritis (2). There has been a resurgence in the incidence and severity of invasive group A *Streptococcus* infections since the mid-1980s in many developed countries (3, 10). It is still unclear whether this increase of invasive group A *Streptococcus* infection is a result of the emergence of particularly virulent clonotypes, the exposure of group A *Streptococcus* to an immunologically naive population, or a combination of both factors. Based on its ability to infect the human host using a variety of different routes, group A *Streptococcus* must be able to adapt to a range of environments (e.g. skin, mucosal surface, blood). Under these circumstances, survival in a variety of changing environments would likely require the expression of distinct subsets of virulence factors. Therefore, genes that are significantly upregulated *in vivo* may be particularly relevant to group A *Streptococcus* disease emergence, severity, and progression. Sera from convalescent patients with invasive group A *Streptococcus* disease and group A *Streptococcus*-immunized mice sera were recently used to screen a partial genomic expression library for group A *Streptococcus* IVI genes. Seventeen genes were found, encoding a variety of IVI products including a putative penicillin-binding protein (Pbp1A), a putative lipoprotein (AtmB), an iron acquisition protein (SiaA/HtsA), and a conserved hypothetical protein homologous to a putative translation initiation factor in *V. vulnificus* (TdcF). A number of these factors are currently being studied for their specific role in pathogenesis (28).

Other prosects

IVIAT is currently being applied to a variety of other microorganisms, including commensal organisms, emerging pathogens, and select agents for bioterrorism such as *Clostridium difficile* (Ryan et al., personal communication) and *Bacillus anthracis* (Ryan et al., personal communication). A recent endeavor focuses on the potential of IVIAT to identify IVI products that may be involved in neurological diseases associated with group A *Streptococcus* infection, such as Tourette syndrome, obsessive compulsive disorder, and pediatric autoimmune neuropsychiatric disorders associated with *Streptococcus* (Handfield and Murphy, unpublished). As will be discussed below, a recent improvement of IVIAT allows the fundamental principals of this strategy to be applied to instances where the host is unable to produce a humoral immune response to an infectious agent.

Change Mediated Antigen Technology (CMAT)

The success of IVIAT in identifying IVI genes of pathogens involved in human infections led to the desire to apply this technology, or one similar to it, to instances where the host does not mount an immune response. From the standpoint of oral infections, it is generally appreciated that periodontitis caused by *Tannerella forsythia* does not lead to a significant

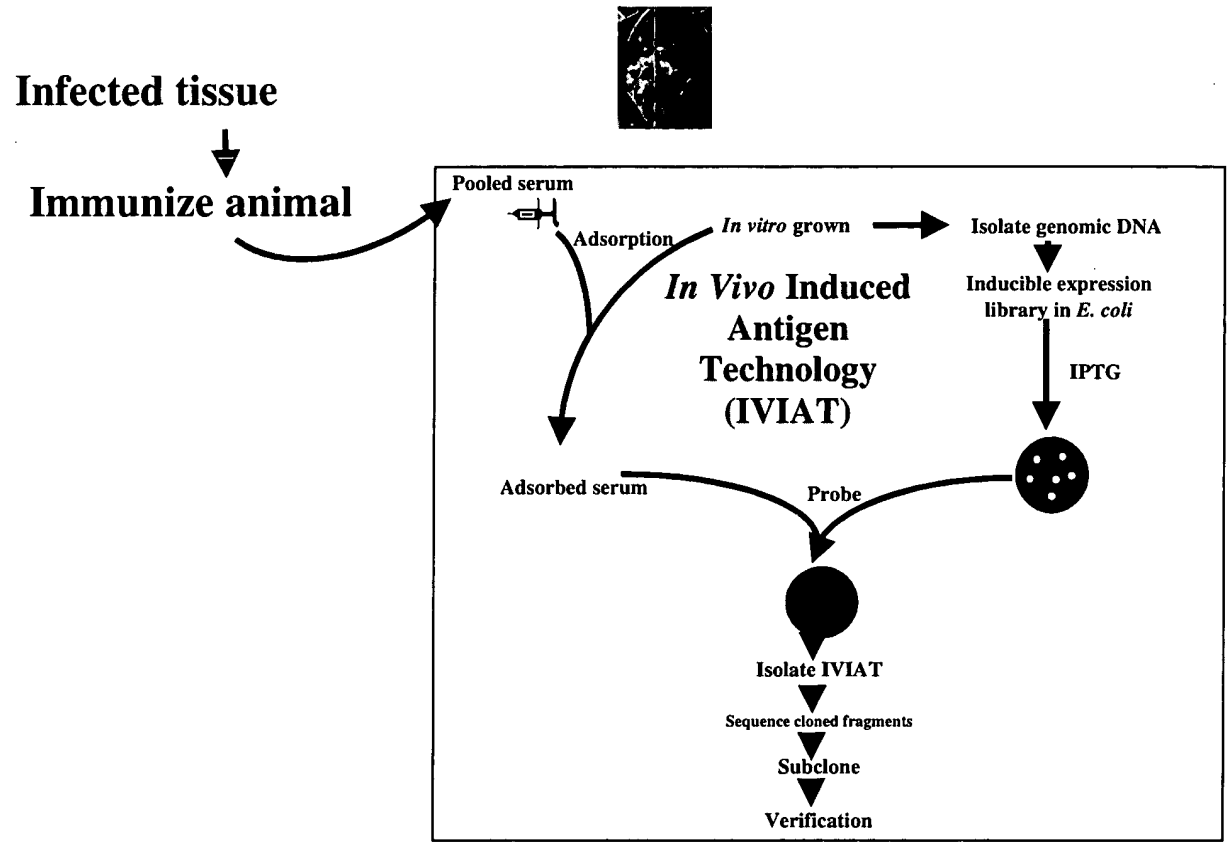

Fig. 2. Change mediated antigen technology (CMAT).

immune response, which would negate the usefulness of IVIAT for the analysis of this microorganism's pathogenic personality. More obviously, plants do not produce antibodies or generate any sort of specific immune response as a result of infection. CMAT provides a route to study these and other systems that cannot be addressed adequately by any methodology currently in existence (Fig. 2).

The key to CMAT is to harvest infected tissue and rapidly preserve the antigens that are present by quick freezing, immersion in a fixative, or other suitable method. Once captured, the antigens present in the sample are used to elicit an immune serum in an appropriate animal. The serum that is recovered can then be adsorbed with cells of the pathogen grown *in vitro* and used in a fashion identical to that described for IVIAT to identify genes of the pathogen that were expressed specifically during the infection process. A particularly interesting feature of CMAT is that the animal used to raise the immune serum will also recognize the infected host tissue as containing foreign antigens, and will produce antibodies directed against them as well as the pathogen. The serum obtained can be adsorbed with healthy tissue from the host and used to probe a genomic expression library of the host in order to identify genes that are expressed in response to the infection. Thus, CMAT can potentially uncover both virulence genes of the pathogen and resistance genes of the host.

Proof of principle for CMAT has been accomplished using bean blight, *Xanthomonas campestris*, infection of the common bean plant *Phaseolus vulgaris* (Fig. 2). Infected leaf tissue was harvested at various time points during the infection cycle and quick frozen. Samples of the infected tissue were combined with adjuvant and used to immunize rabbits. The sera that were obtained were pooled and found to be strongly and broadly reactive with proteins from both the *X. campestris* pathogen and the plant host. Adsorption of the serum with *in vitro* grown *X. campestris* yielded the $CMAT_{pathogen}$ probe that was used to screen a partial *X. campestris* library. Adsorption of the serum with healthy bean plant leaf tissue yielded the $CMAT_{host}$ probe that was used to screen a partial *P. vulgaris* library. In the former case, three putative IVI genes were found. A mutation in one of these genes resulted in a significantly decreased virulence potential, suggesting that CMAT had indeed identified a virulence gene in the plant bacterial pathogen. Reversion studies are currently

underway to confirm this result. In the latter case, two putative IVI genes were isolated. Mutant analysis is not possible in the bean plant, but both of the putative infection-related plant genes are homologs of known resistance factors in other plants (J.D. Hillman et al., unpublished).

The potential applications of CMAT extend beyond infectious diseases. Careful reflection shows that it can be used to identify genes that are specifically expressed when any sort of cell undergoes any sort of change. For example, cells forming a biofilm could be used to immunize an appropriate animal. The resulting serum could then be adsorbed with planktonic cells and used to probe an expression library in order to identify those genes of the microorganism that are specifically expressed during the initiation and progression of biofilm formation. Similarly, cancerous tissue could be used to immunize an appropriate host, and the resulting serum adsorbed with healthy tissue for use in identifying genes that are expressed when the cells undergo neoplastic transformation. A number of CMAT projects are currently underway, and it will be interesting to see what they can accomplish by way of increasing our understanding of a variety of biological processes.

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